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Disease and functional loading effect on the structural conformation and mechanical properties of the mandibular condyle in a transgenic rheumatoid arthritis murine model: an experimental study

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Abstract: AIM The aim of the present study was to investigate the effect of rheumatoid arthritis (RA) and functional loading through diet modification on the structural conformation and the mechanical properties of the mandibular condyle in a transgenic mouse model and compare to healthy littermates. **MATERIALS AND METHODS** Four-week-old hybrid male mice from mixed background CBAxC57BL/6 were used. Four groups of animals were formed consisting of five animals each, either presenting RA (transgenic line hTNF 197), or wild-type (control), half receiving ordinary (hard) diet and half receiving soft diet within each category. Following sacrifice, resin-embedded and metallographically polished condylar specimens were evaluated employing scanning electron microscopy/ Energy dispersive x-ray spectroscopy and also tested for mechanical properties, through Vickers microhardness (HV100) measurements. **RESULTS** The multivariable analysis revealed significantly lower HV100 values for the RA groups after adjusting for diet (= -10; 95% confidence interval: -16, -4; P = 0.001), while functional loading through diet modification did not appear as a significant predictor of the outcome. **CONCLUSIONS** There was evidence of compromised mechanical properties of the mandibular condylar bone for the diseased animals, whereas no association between functional loading and mechanical properties of the condyle could be established.

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Disease and functional loading effect on the structural conformation and mechanical properties of the mandibular condyle in a transgenic rheumatoid arthritis murine model. An experimental study.

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Summary

Aim: The aim of the present study was to investigate the effect of rheumatoid arthritis and functional loading through diet modification on the structural conformation and the mechanical properties of the mandibular condyle in a transgenic mouse model and compare to healthy littermates.

Materials and Methods: Four-week old hybrid male mice from mixed background CBAxC57BL/6 were used. Four groups of animals were formed consisting of five animals each, either presenting rheumatoid arthritis (transgenic line hTNF 197), or wild-type (control), half receiving ordinary (hard) diet and half receiving soft diet within each category. Following sacrifice, resin-embedded and metallographically polished condylar specimens were evaluated employing Scanning Electron Microscopy (SEM)/ Energy dispersive x-ray spectroscopy (EDX) and also tested for mechanical properties, through Vickers micro-hardness (HV100) measurements.

Results: The multivariable analysis revealed significantly lower HV100 values for the rheumatoid arthritis groups after adjusting for diet ($\beta = -10$, 95%CI: -16, -4; $p=0.001$), while functional loading through diet modification did not appear as a significant predictor of the outcome.

Conclusions: There was evidence of compromised mechanical properties of the mandibular condylar bone for the diseased animals, whereas no association between functional loading and mechanical properties of the condyle could be established.

Keywords: rheumatoid arthritis, functional loading, mandibular condyle, hardness, bone mineral status

Introduction

Insights to the subchondral bone of the mandibular condyle have confirmed the anisotropy of the tissue, which largely consists of a number of plate-like and rod-like trabeculae, oriented the first in an anteroposterior and vertical direction, while the second in a transverse direction (1).

Research in the field of mechanical loading has further suggested that the optimal configuration of the cancellous bone trabeculae of the mandibular condyle is the plate-like structure with the plates oriented directly parallel to the sagittal plane. This helps the bone withstand compressive and tensile strains developed along the sagittal axis of the condyle, following the direction of condylar loading (2). However, in case of bone loss, a number of structural changes of the cancellous bone matrix have been identified, namely a decrease in bone density and trabecular thickness (3,4), an increase in anisotropy of the tissue (5) and also an apparent transformation of plate-like trabeculae into rod-like ones. As mechanical loading has been associated with bone morphology and remodeling of the tissue, the latest could also apply when decreased functional loading of the mandibular condyle takes place.

The stress and strain distributions on human bones determine the trabecular morphology of the cancellous bone component and it has been suggested that this morphology is in turn an essential modulator of the mechanical properties of the tissue (6,7). Softening of the ingredients of daily food consumption has been common practice among studies investigating the effects of modified functional loading on the head of the mandibular condyle (8–10).

The degree of bone mineralization is an internal regulator of the structural and biomechanical behavior of the cancellous bone tissue (11,12). Experimental studies have revealed large variations

in the degree of mineralization of condylar bone reflected on the stress and strain distributions within both cortical and trabecular tissue (13).

Of the dominant components of bone matrix is collagen type I, representing approximately 80% of all bone matrix proteins (14), and constituting a cross-linked fibril architecture, being compatible with the plate like shape and orientation of bone hydroxyapatite and controlling the formation and orientation of the crystal apatites (15,16). This conformation, consisting of collagen fibrils integrated within the organic phase of the bone tissue, favors the increase in tensile strength and Young modulus of the fibrils (17,18).

The substantial contribution of collagen network to the bone's structural and mechanical integrity remains questionable in a number of collagen disorders such as rheumatoid arthritis (RA), osteogenesis imperfecta, Marfan syndrome and others. Rheumatoid arthritis is characterized by intra-joint manifestations and synovial membrane inflammation in a symmetric manner (19,20), currently affecting 0.4 to 1 percent of world population (21,22). The most notable manifestations arising from the mandibular condyles are erosions, flattening of the condylar head and osteophytosis (23–25).

To our knowledge, there is currently no study on the combined effects of functional loading and rheumatoid arthritis on the structural conformation and mechanical properties of the subchondral bone of the mandibular condyle. Therefore, the aim of the present study was to assess the effect of rheumatoid arthritis and functional loading through diet modification on the structural and mechanical properties of the mandibular condyle in a transgenic mouse model and compare with healthy littermates. The null hypothesis was that there was no difference between transgenic and healthy littermates with different levels of functional loading as far as structural and mechanical properties of the mandibular condyle are concerned.

Materials and Methods

Twenty hybrid male mice were used. Ten were of transgenic line hTNF 197 (Tg 197- presenting rheumatoid arthritis) from mixed background CBAXC57BL/6 and the rest were healthy littermates CBAXC57BL/6. The sample was obtained from the Biomedical Sciences Research Center “Alexander Fleming” in Vari, Greece (26). The study protocol was approved by the Veterinary Directorate and received the protocol number 1401/07-03-2014, with registration number EL BIO 005, according to the country’s legislation (P.D 56/2013), conforming to the European Directive 2010/63/EU on the protection of vertebrate animals used for scientific purposes.

Four groups of mice were formed. Group 1 [n=5, RA-hard] included transgenic mice and received ordinary (hard) diet throughout the experimental period. Group 2 [n=5, RA-soft] included transgenic line and received a soft diet. Group 3 [n=5, Control-hard] were healthy littermates receiving ordinary (hard) diet and Group 4 [n=5, Control-soft] again healthy littermates receiving a soft diet. The soft diet was produced by the same pellets used for the ordinary food after blending them with water in standardized proportions to achieve porridge like consistency. A single cage was used for each group (ie a total of 4 cages) and allocation to type of diet for the cage as a unit was done through toss of a coin and subsequently alternate intervention assignment for both RA and Control groups. Blinding was not possible for the investigators with respect to type of diet during the experimental phase of the study due to the nature of the interventions.

The 28th day of the animals’ age was determined as day 1 of the experiment. During the experimental period all the animals were fed and watered *ad libitum* and their physiologic growth and development was closely monitored. Experimental living conditions followed National and European legislation and standards, including cages (Tecniplast S.P.A., Italy) and environment with 55% relative humidity, central ventilation (15 air changes/h) and artificial 12-h light-dark cycle. The

total experimental period was set at 4 weeks, during which the animals did not experience any pain or discomfort. At the end of the experiment, the animals were sacrificed after being transiently anaesthetized in an ether chamber and all efforts were made to minimize suffering. The mandibles were separated from the heads and stored in 70% ethanol (right and left hemimandibles with the condyles separately).

Outcome assessors were blinded regarding type of diet during the laboratory procedures, through the use of coding numbers allocated to the study units (ie condylar specimens). However, masking was not possible with regard to health status of the animals as the condyles of the RA mice could easily be macroscopically identified by their shape.

Scanning Electron Microscopy/ Energy dispersive x-ray microanalysis (SEM/EDX)

The right condyle specimens were embedded in epoxy resin in a direction parallel to the longitudinal axis of the condyle through a micro-holder device, thus providing a standardized orientation and allowing the head of the mandibular condyle to be examined systematically. The specimens were ground with 600, 1200 and 4000 grit size silicon carbide papers under water cooling for 2 minutes each, polished with MD-Nap alumina suspensions (Buehler, Lake Bluff, IL, USA) up to 1µm for 3 minutes, in a grinding/polishing machine (Ecomet III, Buehler), and cleaned in an ultrasonic water bath for 5 min. The polished specimens were sputter coated with a thin layer of carbon in a sputter coating unit (SCD 004 Sputter-Coater with OCD 30 attachment; Bal- Tec, Vaduz, Liechtenstein) and examined in a SEM (Quanta 200; FEI, Hillsboro OR, USA), coupled to an energy-dispersive X-ray spectrometer (EDX) equipped with a silicon-drift detector (X/10 SDT, Bruker, Berlin, Germany). Mapping of the whole specimen surface was performed, while surfaces with the highest degree of variability were selected. Compositional backscattered electron imaging (BE) and EDX spectra were collected from each specimen from central and surface regions under the following

conditions: 20 kV accelerating voltage (15 kV for BE images) and 105 μ A beam current, 133×10^{-6} Pa (high vacuum), $98 \mu\text{m} \times 98 \mu\text{m}$ collecting window at 200x magnification and 200s acquisition time. Quantitative analysis was performed against a hydroxyapatite standard with an accuracy of $\pm 3\%$ of the stated values, employing Esprit (Bruker) software.

Microhardness measurements

The same specimens were repolished to eliminate carbon coating and used for Vickers Hardness (HV) measurements using a microhardness tester (HMV2000, Shimadzu, Tokyo, Japan) by focusing on trabecular pattern and applying 100g load for 15 s contact time. At least 3 indentations were performed per specimen and the HV100 was obtained after averaging the values within each specimen.

Statistical analysis

No a-priori sample calculation was conducted. Descriptive statistics (median, range, minimum/maximum values) were used to present the results of the study. Weight changes were calculated and compared through 2-way ANOVA. Univariable and multivariable linear regression was performed, in order to assess the effects of the disease (rheumatoid arthritis) and type of loading (hard diet/soft diet) (predictor variables) on the outcomes of interest. Standard errors (SE) were calculated using the bootstrap method with 500 replications (bootstrapping regression model). Model fit was checked through Kernel density residual plot.

Data analyst was blinded regarding specimen identity and group allocation through the use of coding numbers for all collected data.

The level of significance was pre-specified at $\alpha=0.05$. All statistical analyses were conducted with STATA® version 12.1 software (Stata Corporation, College Station, Texas, USA).

Results

Baseline animal weights were as follows: Group 1 (RA-hard, median 18g, range: 17-21), Group 2 (RA-soft, median 17g, range: 16-19), Group 3 (Control-hard, median 19g, range: 17-22), Group 4 (Control-soft, median 20g, range: 18-22). Weight changes of the animals throughout the experimental period were comparable across the four groups ($p=0.16$). Median weight increase for Group 1 (RA-hard) was 9g (min: 7g, max: 10g) and for Group 2 (RA-soft) 9g (min: 8, max: 10). Similarly, for the control groups the following weight changes (increases) were recorded: Group 3 (Control-hard): median 9g (min: 8, max: 10) and Group 4 (Control-soft): median 10g (min: 9, max: 11).

Scanning Electron Microscopy/ Energy dispersive x-ray microanalysis (SEM/ EDX)

Figure 1 demonstrates backscattered electron images from representative areas analyzed among the animal groups tested, along with representative EDX spectra (Figure 2). Table 1 shows the results of elemental atomic ratios recorded for the four groups. Ca/P and N/Ca ratios were comparable along groups with regard to both core and surface related spectra. Mg/Ca along with S/Ca ratios values were kept to minimum. On an exploratory basis, spot surface spectra revealed evidence of increased sulfur (S) concentrations (at%) in the control animals (S: 0.18 at%), while there was no such finding for the RA groups (S: 0.02 at%).

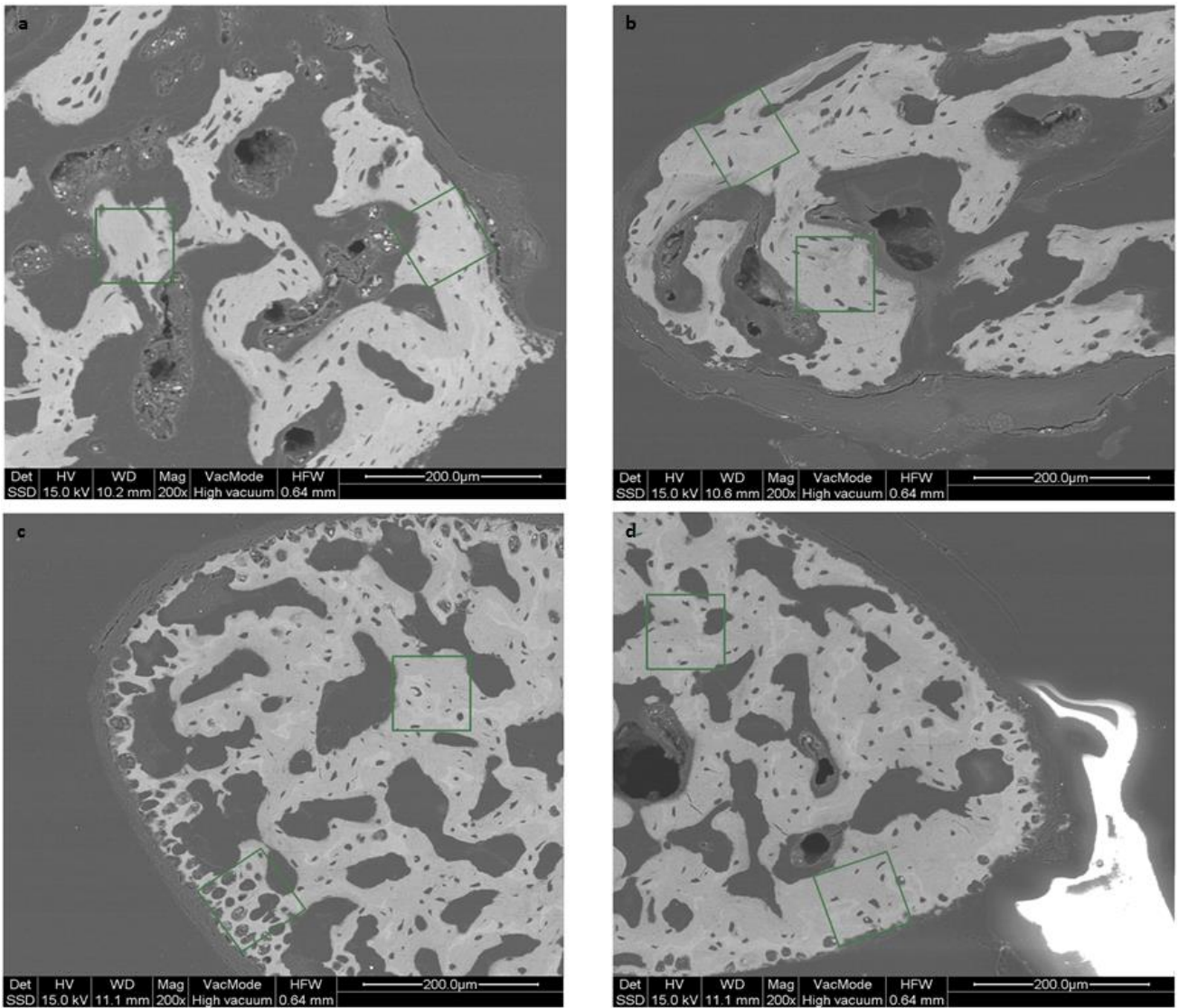


Figure 1. Representative backscattered electron (BE) images of specimens (horizontal condylar head sections showing hemispheres of the specimens). The areas with green margins correspond to the windows used for EDX spectra acquisition. (a) RA-hard group, (b) RA-soft group, (c) Control-hard group, (d) Control-soft group.

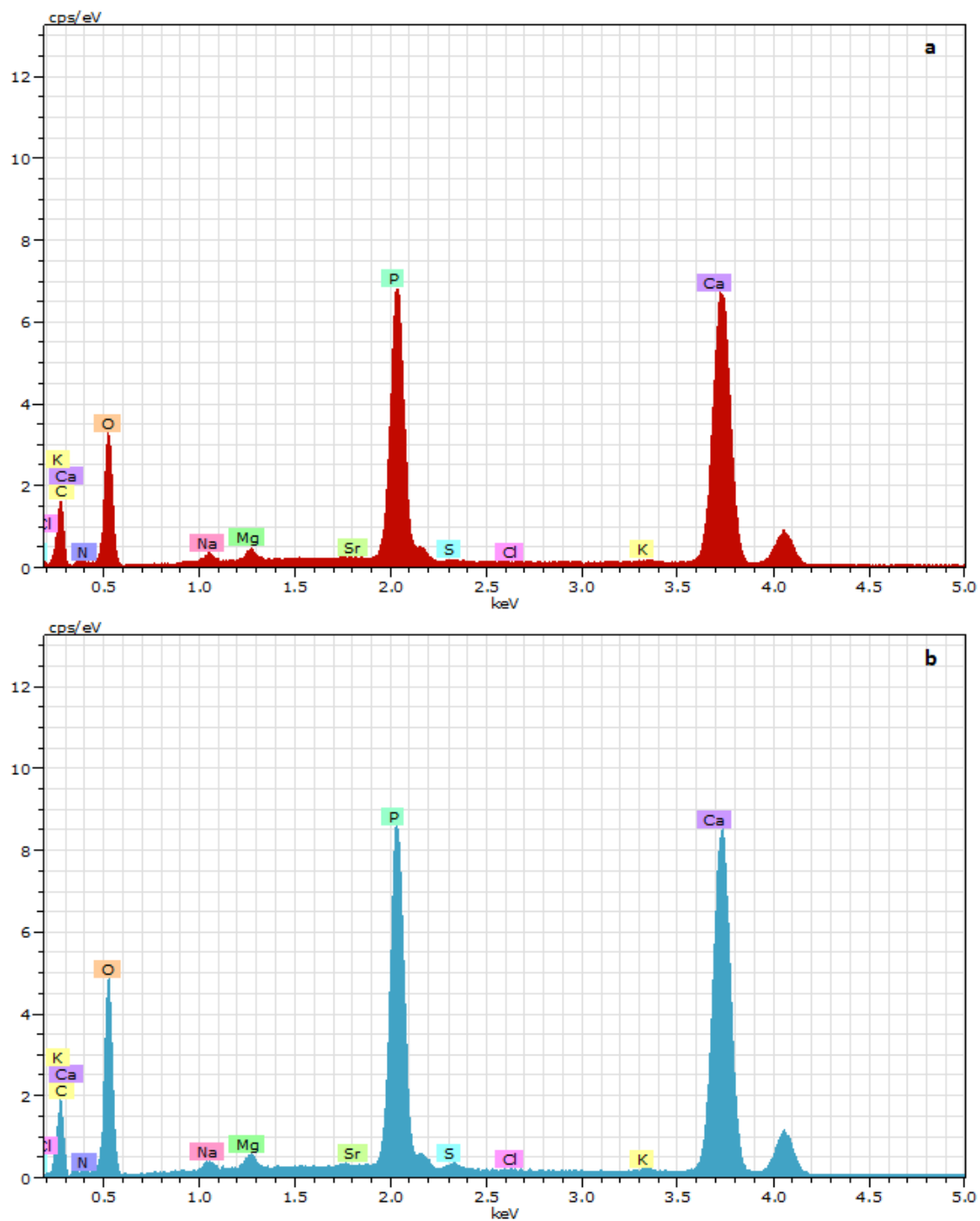


Figure 2. Representative EDX core spectra with atomic concentrations (at%). (a) RA-hard group, (b) Control-hard group.

Table 1. Atomic ratios according to EDX analysis.

Spectra Acquisition		RA-hard	RA-soft	Control-hard	Control-soft
<i>Core</i>	Ca/P	1.65	1.80	1.78	1.93
	N/Ca	0.40	0.58	0.28	0.52
	Mg/Ca	0.03	0.03	0.03	0.03
	S/Ca	0.004	0.002	0.01	0.00
<i>Surface</i>	Ca/P	1.76	1.65	1.64	1.81
	N/Ca	0.56	0.43	0.35	0.54
	Mg/Ca	0.03	0.03	0.05	0.03
	S/Ca	0.003	0.009	0.005	0.002

Micro-hardness measurements

Median hardness values per group were as follows: group 1 [RA-hard], HV100 median=41 (range: 33-49); group 2 [RA-soft], HV100 median=42 (range: 25-47); group 3 [Control-hard], HV100 median=53 (range: 44-57); group 4 [Control-soft], HV100 median=46 (range: 41-53) (Figure 3).

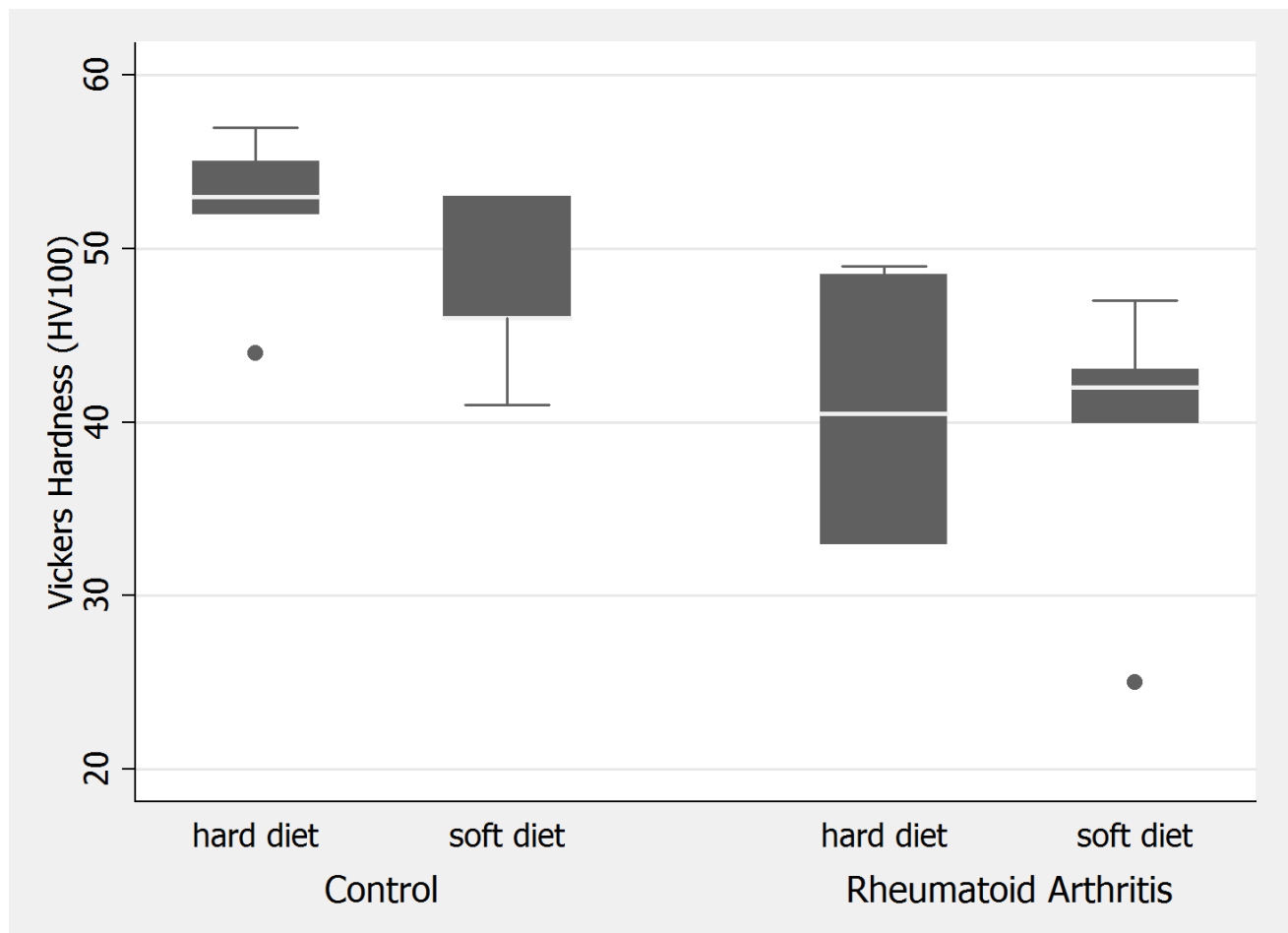


Figure 3. Boxplots representing Vickers Hardness (HV₁₀₀) measurements across groups.

The multivariable analysis revealed significantly lower HV100 values for the RA groups compared to control after adjusting for diet ($\beta = -10$, 95%CI: -16, -4; $p=0.001$) (Table 2, Figure 4).

Table 2. Univariable and multivariable linear regression with observed coefficients and 95% confidence Intervals (CIs), for Vickers Hardness (HV₁₀₀). Standard errors (SE) were calculated using the bootstrap method with 500 replications.

	Category	Univariable Analysis			Multivariable Analysis		
		Observed coefficient (β)	95% CI	p-value	Observed coefficient (β)	95% CI	p-value
Disease	<i>No (control)</i>	Reference			Reference		
	<i>Yes (rheumatoid arthritis)</i>	-10	-16, -4	0.001	-10	-16, -4	0.001
Diet	<i>Hard (standard)</i>	Reference			Reference		
	<i>Soft</i>	-4	-11, 4	0.35	-3	-9, 3	0.32

Interaction term (disease*diet) is non-significant (p=0.66).

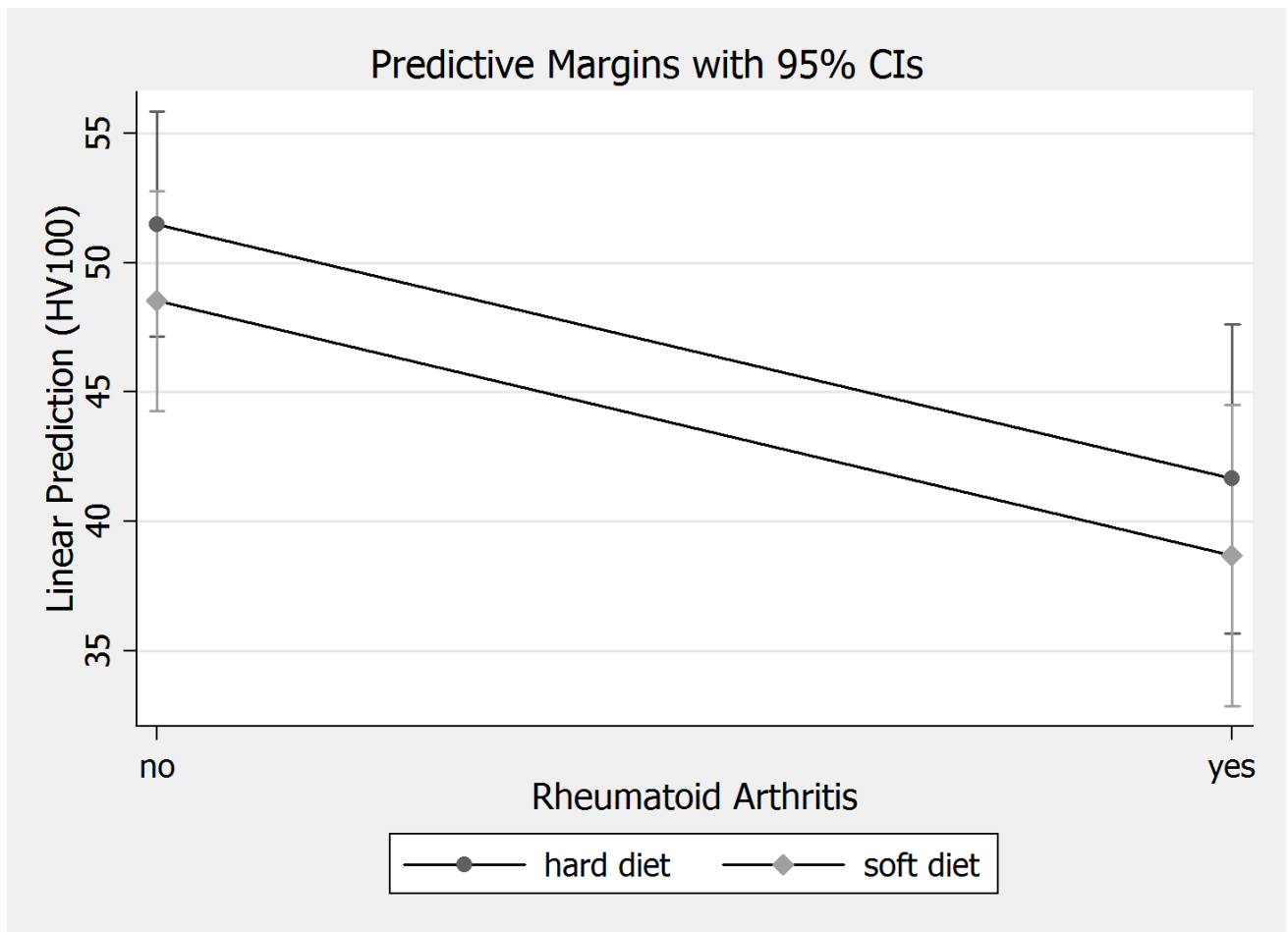


Figure 4. Adjusted predictive margins with 95% CIs for the effect of disease and functional loading (diet) on Vickers Hardness (HV₁₀₀).

Representative indentations demonstrating load application on condylar bone specimens are shown in Figure 5.

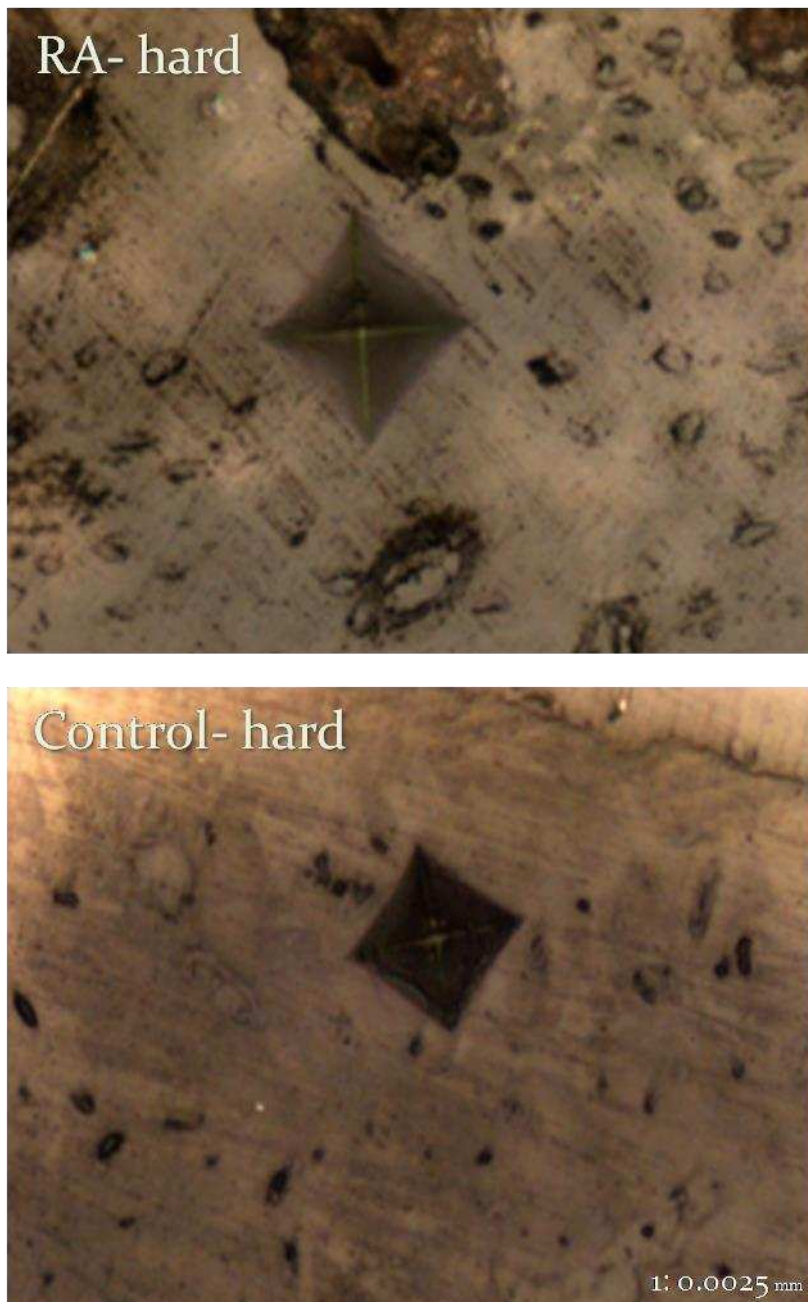


Figure 5. Representative indentations on condylar bone specimens for RA and Control groups.

Discussion

Notwithstanding a number of animal studies about the effect of functional loading on the mandibular condyle, there is currently no evidence regarding the mechanical behavior and structural conformation of the condylar bone under compromised status of collagen integrity as is the case with rheumatoid arthritis disease. The interplay with differential functional loading was also assessed. The results of the present study revealed evidence of compromised mechanical properties in both RA groups, thus leading to rejection of the null hypothesis with regard to no effect of the disease; however, functional loading through diet modification did not appear as a significant predictor of bone properties of the mandibular condyle in either RA or control animals.

The murine strain used in the present study has been considered a well-established model for research in the field of rheumatoid arthritis as it has been generated through the intermediation of a recombinant gene construct with the potential to express 3' modified hTNF transgenes resulting in the development of a chronic inflammatory polyarthritis and involving both major and smaller joints; however, it is the first time the temporomandibular joint (TMJ) of the model is being studied (26–28)(26).

SEM/EDX findings did not reveal evidence of compromised bone mineral status of the condyles in the testing groups, while increased sulfur (S) concentrations close to the surface were detected in the control groups and might be related to the presence of cartilage tracks. Sulfate findings close to the peripheral zone of the control specimens are presumably attributed to proteoglycan aggregate macromolecules bearing glycosaminoglycan chains (GAG), mainly represented by chondroitin and keratan sulfate chains (29). Aggrecan is of the most abundant extracellular matrix macromolecules of the articular cartilage, providing structural integrity and functional capacity to joint cartilage as well as its ability to resist compressive loading (30).

The disruption of the characteristic structural integrity of the condylar bone consisting of collagen fibrils embedded within the hydroxyapatite network as a result of rheumatoid arthritis has appeared to impact on the mechanical properties of the tissue in the RA animals irrespective of the type of dietary loading. It has been suggested that woven-like bone, which is characterized by unorganized collagen fibrils, presents compromised mechanical properties compared to physiologic cancellous bone, indicating the significance of collagen content in the biomechanical characterization of the bone matrix (31). It is therefore likely that the effect of rheumatoid arthritis on the mechanical properties of the subcartilage condylar bone is most probably attributed to the internal derangement of the collagen matrix, rather than to the mineral phase of the tissue.

Research in the field of biochemical properties of long bones through the use of spectroscopy (fourier transform infrared (FTIR)/ Raman) and oriented to osteoporosis/experimental arthritis models has confirmed either non-detectable, or small variations on bone mineral-phase compositional metrics (ie. mineral to matrix ratio) between diseased and control specimens (32,33).

The selection of loading force was based on previous research on indentation testing on bone surfaces (15). Average values of hardness measurements through at least 3 indentations per specimen were employed on the basis of the nature of the bone entity and in order to eliminate tissue variability effects, as bone mechanical properties are considered site-specific due to the anisotropy of the tissue (34). It was not always possible to acquire more than 3 readings per specimen as there was need to account for sufficient distance among the indentation-indentation or indentation-surface interface in order to allow for tissue relaxation after loading application to take place.

Previous research in the field of altered functional loading, in healthy growing animals has revealed reduced bone mineral density and trabecular bone volume and thickness of the mandibular

alveolar process associated with decreased masticatory loading (35). With regard to mandibular condyle, Kiliaridis *et al* (9) demonstrated decreased gross condylar dimensions in growing rats receiving soft diet, whereas, cartilage thickness followed a spatial distribution, with animals in the soft diet group presenting thinner anterior and thicker posterior parts of the cartilage.

The effect of rheumatoid arthritis on bone biomechanics has recently been recorded in animal research using the same transgenic murine model and through the implementation of torsional and compressive mechanical testing on the long bone diaphysis (tibia) and flat bone (vertebral bodies) respectively. Decreased bone strength and increased ductility was associated with RA (36).

However, currently there is no evidence on the interrelationship between a collagen degenerating disease such as rheumatoid arthritis and functional loading on the condylar bone. In addition, there is lack of similar research regarding the effects of RA on the mechanical profile of the mandibular condyle, thus precluding any direct comparison with other studies.

Limitations of this study may pertain to the inherent issues affected by food consumption and the subsequent condylar loading related to the murine models, as there was no attempt to directly measure the exact daily food consumption. However, food was renewed daily in a standardized manner/proportions and within identical food trays, while there were no significant weight-change differences detected across groups within the experimental period. Another limitation of the present study may be an insufficient power to detect small effect size differences due to the small number of animals per group. However, adoption of ARRIVE guidelines (37) on animal research and particularly following of the "Three Rs" scheme (ie. Replacement, Reduction, Refinement) (37) has led us to recruit as few animals as possible to obtain sufficient levels of information. This, in conjunction with the use of the bootstrap method with 500 replications built-in the regression

model for the statistical analysis of the data may have most probably accounted for the small sample size.

On the same basis with the aforementioned "Reduction" regarding the "Three Rs" guiding principles for more ethical use of animals in experimental research, "Replacement" could not have been applicable in the present study as the transgenic strain used has been considered a well-established model for research purposes presenting chronic inflammatory polyarthritis and involving both major and smaller joints (26). No other experimental animal model could have been considered more appropriate for the present research. Moreover, none of the animals experienced any pain or discomfort throughout the experimental period, while upon sacrifice all actions were taken to minimize suffering though transient anaesthetization in an ether chamber.

Currently, more sophisticated methods such as nanoindentation have been developed to map the elastic modulus and hardness at the level of individual osteons for the cortical bone, or at the level of individual trabeculae for the trabecular bone (38). However, as this was the first time mechanical properties of the subchondral condylar bone were ever examined within the spectrum of a collagen disease, a more standard engineering test method was employed to detect differences in hardness between physiologic and disrupted bone structure (39).

Extrapolation of the results of the present study to human or clinical investigations should be considered with caution. The selection of the transgenic animal model used in this study has postulated a number of significant advantages over the viable alternative of an induced arthritis model and it has been selected on the basis of the following (26): the transgenic strain developed chronic polyarthritis comparable to human rheumatoid arthritis, complete phenotypic penetrance and mandibular condyles symmetrically affected.

Conclusions

In conclusion, the present study aimed to assess for the first time the effects of a collagen degenerative disease and functional loading on the mandibular condylar bone. Mechanical testing to the mandibular condyles revealed lower HV₁₀₀ values for the animals presenting RA, while there was no evidence of detectable differences in the mineral phase of the subcartilage condylar bone among the testing groups. In addition, no correlation between functional loading and structural conformation or mechanical properties of the mandibular condyles could be established within the time span assessed.

Competing Interests

The authors have no competing interests to declare

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